KINETIC AND SPECTROSCOPIC EVIDENCE FOR DIFFERENT FORMS OF FERRIC CYTOCHROME *c* AT VERY LOW IONIC STRENGTH AND NEUTRAL pH

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1. Introduction

A proper description of the reactions of ferric cytochrome *c* with ionic reductants requires a study of the effects of ionic strength on these reactions. During the course of such experiments, it was found that in neutral solutions of very low ionic strength the reduction of ferric cytochrome *c* with ascorbate followed biphasic kinetics. These results are reported herein together with preliminary evidence for a possible conformation equilibrium of the protein in solutions of very low ionic strength.

2. Materials

Horse heart cytochrome *c* (Sigma Chem. Co., Type VI) was percolated through Bio-Gel P4 columns equilibrated with Tris–cacodylate buffer, 0.002 M, pH 7.2. Other reagents were of analytical grade.

3. Methods

Spectra and kinetic measurements were recorded on a Cary 118-C instrument; fast kinetic experiments were done with a stopped-flow attachment in an Aminco-Chance dual-wavelength spectrophotometer at 416–430 nm.

4. Results

Figure 1 shows kinetic measurements of the reduction of 5×10^{-6} M ferricytochrome *c* by 2×10^{-3} M Na-ascorbate, at pH 7.2, in Tris–cacodylate buffer, 0.002 M. Without added salt (for this solution the contribution of protein, buffer and reductant give $I = 0.004$) the reduction was clearly biphasic; in the presence of 0.1 M NaCl, $I = 0.104$, monophasic kinetics were observed (fig.1). Analysis of the biphasic kinetics permits to distinguish between the fast- and slow-phases. While the rate constant for the slow-phase did not depend on ascorbate concentration up to 0.0025 M, the rate constant for the fast reaction increased with ascorbate concentration (fig.2). A difference spectrophotometric titration of ferricytochrome *c* at various NaCl concentrations measured against the protein in 0.002 M Tris–cacodylate buffer showed small but definite shifts in the Soret region (fig.3).

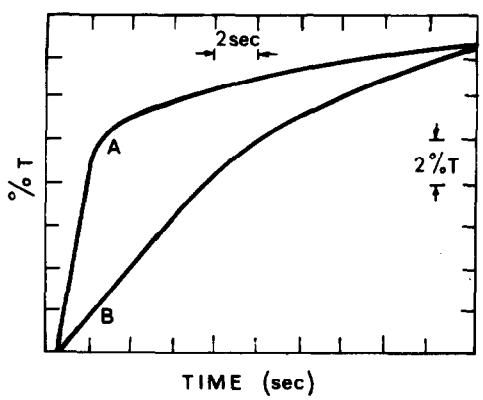

Fig.1. Reduction of 5×10^{-6} M ferricytochrome *c* with 2×10^{-3} M Na-ascorbate at pH 7.2, Tris–cacodylate buffer 0.002 M. (A) Without added salt. (B) With NaCl 0.1 M. Kinetics were followed in a double-beam spectrophotometer at 416–430 nm, 25°C.

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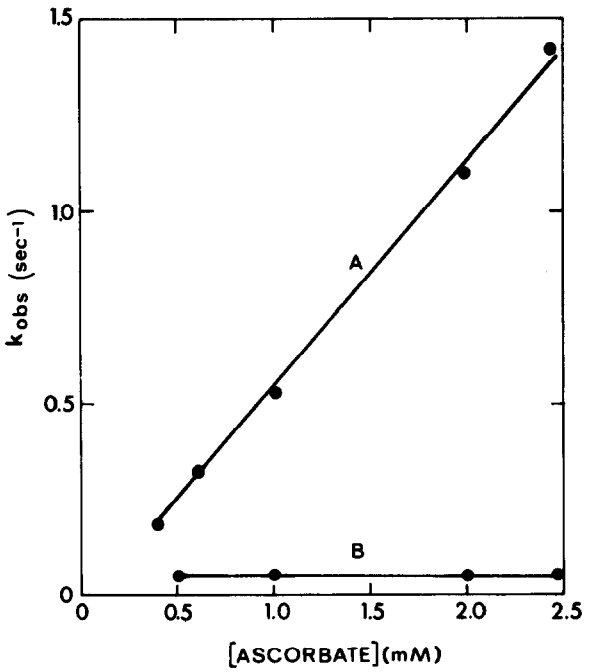

Fig.2. Second-order plot for the reduction of 5×10^{-6} M ferricytochrome *c* with Na-ascorbate at 25°C, 416–430 nm in 0.002 M Tris–cacodylate buffer, pH 7.2. (A) For the rapid-(initial)-phase. (B) For the slow-phase.

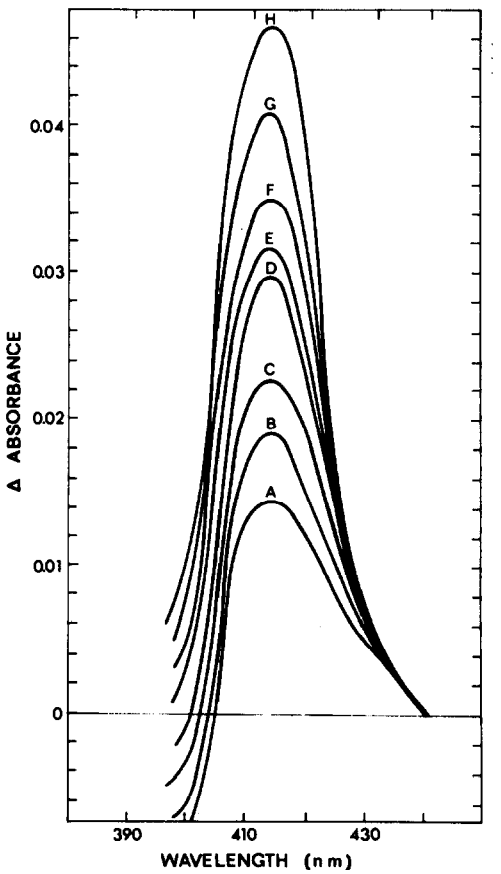

Fig.3. Difference spectroscopic titration of 10^{-5} M ferricytochrome *c* with NaCl, at pH 7.2, Tris–cacodylate buffer, 0.002 M, 25°C. NaCl concentrations were increased by successive and constant additions of 0.02 M NaCl (A–H total change of salt concentration from 0.02–0.15 M respectively).

From these difference spectra and the spectrum of ferricytochrome *c* in 0.15 M NaCl, the spectrum of the Soret band of salt-free ferric cytochrome *c* was estimated; the maximum wavelength was found at 408 nm with $\epsilon = 104.2$ mM^{-1}. This should be compared with the corresponding values for the enzyme in 0.15 M NaCl, which are: $\lambda_{max} = 410$ nm and $\epsilon = 106.1$ mM^{-1} [1].

5. Discussion

The simplest hypothesis that explains the kinetic experiments reported above is that in the absence of salts, at $I \rightarrow 0$, ferric cytochrome *c* is present in at least two different conformations: one that reacts rapidly with ascorbate, and another that reacts very slowly or not at all. The rapidly-reacting form accounts for 80–90% of the total protein. The slow-phase of the reaction corresponds to the conversion of the second form into the first, and is therefore independent of reductant concentration. The addition of salts shifts the conformation equilibrium towards the fast-reducible form and, through ionic strength effects, decreases the observed rate of the bimolecular reduction of the fast-reacting form.

The spectroscopic changes detected in the Soret region (fig.3), are in keeping with a salt-dependent conformation equilibrium. Moreover, similar salt additions increased slightly the absorbance of the entire near-infrared region but without causing a definite change in the 695 nm band. Attempts to detect conformation changes by other techniques such as fluorescence and circular dichroism gave preliminary indications that support this interpretation, but the changes

observed were small and require further experimental elaboration. The small extent of the observed changes recalls the similar minor effects on the Soret bands of other hemoproteins brought about by solvent perturbation [2]. This and the persistence of the 695 nm band in all the forms indicates that the conformers differ by rather small structural rearrangements, which makes even more intriguing the fact that they differ so notably in their reactivity with an electron-donor. Preliminary studies on the rate-constant of the fast- and slow-phases indicate that, as expected from purely electrostatic considerations, the former are strongly diminished by increasing ionic strength, while the latter are practically unaffected. The outcome of this situation is that the biphasic nature of the reaction in the absence of salt is lost as the ionic strength reaches a certain finite-value. It should be pointed out that, although the biphasic nature of the reaction in low salt recalls similar kinetic effects observed at alkaline pH [3], the two situations cannot be compared before a thorough study of the influence of salts on the reaction at alkaline pH is performed. The fact that biphasic kinetics of ferricytochrome *c* reduction, with ascorbate at neutral pH, were not observed by other authors [3] is obviously due to their use of salt concentration at which the reaction is already monophasic (fig.1).

Acknowledgements

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